Title: Late life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters

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Abstract

This study examined late life effects of perinatal exposure of rats to a mixture of endocrine disrupting contaminants. Four groups of 14 time-mated Wistar rats were exposed by gavage from gestation day 7 to pup day 22 to a mixture of 13 anti-androgenic and estrogenic chemicals including phthalates, pesticides, UV-filters, bisphenol A, parabens and the drug paracetamol. The groups received vehicle (control), a mixture of all 13 chemicals at 150-times (TotalMix150) or 450-times (TotalMix450) high end human exposure, or a 450-times mixture of 9 predominantly anti-androgenic chemicals (AAMix450). Onset of puberty and estrous cyclicity at 9 and 12 months of age was assessed. Significantly fewer females showed regular estrus cyclicity at 12 months of age in the TotalMix450 and AAMix450 groups compared to controls. In 19 months old male offspring, epididymal sperm counts were lower than controls and in ventral prostate, an over-representation of findings related to hyperplasia was observed in exposed groups compared to controls particularly in the group dosed with anti-androgens. A higher incidence of pituitary adenoma at 19 months of age was found in males and females in the AAMix450 group. Developmental exposure of rats to the highest dose of a human relevant mixture of endocrine disrupters induced adverse effects late in life manifested as earlier female reproductive senescence, reduced sperm counts, higher score for prostate atypical hyperplasia and higher incidence of pituitary tumors. These delayed effects highlight the need for further studies on the role of endocrine disrupters in hormone-related disorders in aging humans.
Introduction

Exposure to endocrine disrupting chemicals (EDCs) during development is suggested to contribute to a rise in various types of reproductive problems in humans (Skakkebaek et al. 2001, Crain et al. 2008). It is generally accepted that a lag between the time of exposure to EDCs and the manifestation of a disorder can occur (Diamanti-Kandarakis et al. 2009), and in experimental animals adverse reproductive effects after developmental exposure to mixtures of environmental EDCs have been observed both shortly after birth, in puberty and in young adulthood (Hass et al. 2007, 2012, Christiansen et al. 2012, Jacobsen et al. 2012). However, there are indications that early exposure to EDCs may also induce adverse effects that manifest themselves late in life, such as premature reproductive senescence (estropause/menopause) Gore et al. 2011), although the analysis of late life effects has received considerably less attention than the effects of EDCs that become apparent early in life..

In humans, the risk of developing diabetes, cardiovascular disorders or cancer increases with age. Prostate cancer has one of the highest prevalences in humans, and even though the basis for abnormal prostatic growth is not completely understood, both androgens and estrogens are thought to play a role (Bostwick et al. 2004). In rats exposed neonatally to the endocrine disrupter bisphenol A (BPA) and implanted with estradiol and testosterone capsules as adults, an increased susceptibility to preneoplastic lesions in the prostate has been seen (Ho et al. 2006, Prins et al. 2011). In women, estrogen exposure is thought to be a risk factor for breast cancer development (Travis & Key 2003) and exposure to diethylstilbestrol (DES) during development has been shown to increase the risk for breast cancer (Palmer et al. 2006). Developmental exposure to EDCs may therefore play a role in the induction of both prostate and breast cancer later in life. The regulatory guidelines currently used for the risk assessment of chemicals in e.g. the Organisation for Economic Co-operation and Development (OECD) do not include investigations of effects in old age after
developmental exposure (OECD 2001, OECD 2012). Consequently such effects are rarely examined and can be overlooked, and studies evaluating effects in aging offspring are needed.

We have performed an in vivo rat study with a mixture of 13 EDCs (Table 1), modeled on information about environmental exposure in humans, and have investigated effects in aging animals. The rationale behind the chemical selection, a detailed description of the test compounds, the assumptions made when going from high end human estimates to the adjusted exposures that were used in the mixture and the calculation of the mixture dose, are presented in Christiansen et al. (2012). Briefly, eight of the selected chemicals were considered to have predominantly anti-androgenic properties. These included the two phthalates di-n-butyl phthalate (DBP), and di-(2-ethylhexyl) phthalate (DEHP), the five pesticides vinclozolin, prochloraz, procymidone, linuron, epoxiconazole, and the pesticide metabolite dichlorodiphenyl-dichloroethylene (p,p’-DDE). The mixture also contained four predominantly estrogenic substances, the 2 UV-filters octyl methoxycinnamate (OMC) and 4-methyl-benzylidene camphor (4-MBC), the phenolic compound BPA, and the preservative butyl paraben. Finally, the mixture included the analgesic drug paracetamol, which has previously been shown to act as an anti-androgen (Kristensen et al. 2012).

Doses of 150 (TotalMix150) and 450 (TotalMix450) times high end human exposures were chosen for the study. Additionally, a mixture consisting of only the nine predominantly anti-androgenic compounds including paracetamol was included at a dose equivalent to 450-fold the high end human exposures (AAMix450) (Table 1). Doses of 150 and 450 times high end human exposures may appear high at first glance, but they are of relevance, considering the toxicokinetic differences between rats and humans. It is widely accepted that higher doses need to be administered to rats to achieve comparable effects, and this is the basis for using default uncertainty factors of 100 for the extrapolation of effects in rats to humans. These exposures caused dose related effects in pre-pubertal offspring on early markers of sexual differentiation, including increased nipple retention.
and altered weights of some reproductive organs (Christiansen et al. 2012). The current paper presents how developmental exposure of rats to this human relevant mixture of endocrine disrupting environmental contaminants leads to increased risk for adverse effects later in life.

Materials and methods

Animals and exposure

The animal study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee of the National Food Institute at the Technical University of Denmark.

In this exploratory in vivo study, 56 time-mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) three of pregnancy. The study was performed using two blocks of 28 dams separated by one week, and with equal distribution of dose groups.

The animals were housed in pairs until GD 17, and alone thereafter under standard conditions described in detail in Christiansen et al. (2012). Polycarbonate cages (15x27x43 cm) with wood chip bedding, nesting material and plastic shelters were used, and the animals were placed in an animal room with controlled environmental conditions with a 12 hour light-dark cycle starting at 9 PM, and controlled humidity, temperature and ventilation. All animals were fed on a standard diet with ALTROMIN 1314 (soy- and alfalfa-free, ALTROMIN GmbH, Lage, Germany). Acidified tap water in polycarbonate bottles was provided ad libitum.

On GD 4, the dams were randomized into four groups of 14 animals with similar body weight distributions. Dams were dosed daily by oral gavage from GD 7 to the day before expected birth.
(GD 21) and again after birth from post natal day 1 to 22 with either vehicle (control, corn oil (VWR - Bie & Berntsen, Herlev, Denmark) or one of three mixtures. These included the total mixture at 150 (TotalMix150) or 450 (TotalMix450) times the estimated high end human exposure and a mixture including only the 9 chemicals with predominantly anti-androgen mode of action including paracetamol (AAmix450). Mixture composition and dose levels can be seen in Table 1. Paracetamol was only added to the mixture groups between GD 13 and 19. Corn oil was used both as a control compound and as a vehicle. The day when a vaginal plug was detectable was designated as GD 1 and the expected day of delivery (GD 23) was designated as pup day (PD) 1.

After weaning of the offspring (PD 22), two males and two females per litter, if possible, were randomly designated for examinations later in life. Weaned rats were caged in pairs of same sex and exposure.

Onset of puberty

Onset of puberty was assessed by determining day of vaginal opening or the day of balano-preputial separation in weaned female and male offspring, respectively. Registrations were performed daily in females from PD 27 until vaginal opening was detected in all animals. Males were examined daily from PD 39 until the last male was positive. Age and body weight of the rats were recorded on the day where vaginal opening and balano-preputial separation was first observed.

Estrous cyclicity

Vaginal smears were collected every day between 8 and 10 AM, for 21 consecutive days starting when the female offspring were around 9 months-old, and again in rats about 12 months-old. The smear samples were air-dried, fixed in 96% ethanol, stained with Gill’s hematoxylin, Orange G6 and eosin-azure 50 (VWR - Bie & Berntsen, Herlev, Denmark) according to the adapted Papanicolaou (PAP stain) procedure (Hubscher et al. 2005), mounted in Eukit (VWR - Bie &
Berntsen, Herlev, Denmark) and examined by light microscopy by an investigator blinded to the exposure groups. Stages were recognized in terms of the presence, absence or proportional numbers of epithelial cells, cornified cells and leukocytes as described in OECD guidance document 106 and by Goldman and co-workers (Goldman et al. 2007, OECD 2009) and were classified as estrus, metestrus, diestrus or proestrus or transitions between stages.

The animals were categorized as either being regularly cycling (cycles lasting 4 to 5 days), or as being irregularly cycling which was defined as having cycles lasting less than 4 days or more than 5 days (Cooper & Goldman 1999). Furthermore, episodes of 3–4 consecutive days of vaginal estrus and 4–5 days of diestrus were considered extended.

**Necropsy male and female offspring at 19 months of age**

At around 19 months of age, one male and one female offspring from different litters (Table 2a and 2b) were weighed, anesthetized in CO\(_2\)/O\(_2\) and decapitated. Trunk blood was collected in sodium-heparine coated tubes and plasma samples were stored at -80°C after centrifugation for 10 min, 4000 rpm at 4°C. Males were evaluated for testicular descent, cleft phallus, hypospadias and alopecia in the perineal area as described by Christiansen et al. (2008). Rats were necropsied, all relevant gross lesions were described, and tissue samples from lesions were fixed in formalin. Uterus, alternately left or right ovary, testes, seminal vesicle, ventral prostate, alternately left or right epididymis, and liver were removed and weighed. From female offspring the uterus, alternately left or right ovary, the fourth abdominal mammary gland (including lymph node) and the pituitary glands with gross lesions were fixed in formalin for histopathological examination. From male offspring, alternately left or right testicles were fixed in Bouin’s, and seminal vesicle, left or right lobe of the ventral prostate, alternately left or right caput epididymis and pituitary glands were fixed in formalin for histopathological examination. Alternately left or right cauda epididymis
including 1 cm of ductus deferens was frozen in liquid nitrogen and stored at -80°C for sperm count analysis.

**Histopathology**

Fixed tissue samples were routinely processed, embedded in paraffin, sectioned (3µm), stained with hematoxylin and eosin and examined blindly to treatment groups. One cross-section of one uterine horn and one section of the ovary were evaluated from each female. In ovarian sections the presence of corpora lutea, nonatretic and atretic antral follicles and follicular cysts was noted. The stage of the estrous cycle was determined by evaluation of uterine and ovarian histology. Female mammary glands were examined for dilation of ducts and secretory material in the ducts. In one section of the ventral prostate from each male the following were scored: Epithelial atrophy (score 1-3 according to the proportion of acini lined by atrophic epithelium), epithelial atypical hyperplasia (no acini with atypical hyperplasia (score 0), few acini with atypical hyperplasia (score 1), <50% of acini with atypical hyperplasia (score 2), ≥50% of acini with atypical hyperplasia (score 3)), interstitial inflammation (score 0-4 relative to severity of inflammation) and luminal concretion (score 1-3 according to the amount of concretions and the number of acini affected). Furthermore, the presence of cribriform patterns in areas with acinar cell hyperplasia was noted. Testes were examined with an emphasis on effects that can be expected in aging rats or following exposure to anti-androgens including spermatid retention, tubular dilation, degeneration of germ cells at specific stages, and Leydig cell hyperplasia or adenoma. In the epididymal caput, examination focused on the presence of sloughed testicular cells in the epididymal lumen, the amount of spermatids, vacuolization and degeneration in the epithelium of the main caput segment, disorganization of the epithelium in the initial segment, and interstitial inflammation. At least one section of all excised female and male pituitary glands was examined histologically with focus on presence of nodular hyperplasia and adenoma in pars distalis (MacKenzie & Boorman, 1990).
Sperm count analysis

For sperm count analysis, samples were analysed using computer assisted sperm analysis (CASA). The cauda epididymis was thawed, weighed and prepared as described by Jarfelt et al. (2005), and samples were analyzed using a 10 × UV fluorescent objective and IDENT OPTIONS on the CASA. Ten fields were analysed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda. The sperm count was performed in two rounds. All control and TotalMix450 samples were analysed in the first round and hereafter samples from the TotalMix150 group and AAMix450 group were analysed, i.e. this analysis was performed without simultaneous analysis of samples from the control group. Furthermore, due to a technical error during preparation of the samples, it was only possible to obtain results from three animals, representing two litters, from the TotalMix150 group.

Hormone levels

Testosterone and inhibin B levels were analysed in the plasma from male rats. Testosterone was extracted from the plasma on IST Isolute C18 SPE columns as previously described (Vinggaard et al. 2005) and samples were resuspended in heptane. The hormone levels were analysed using Delfia time-resolved fluorescence kits (PerkinElmer Life Sciences, Turku, Finland), and measured by using a Wallac Victor 1420 multilable counter (PerkinElmer Life Sciences, Turku, Finland). Inhibin B levels were measured using an Elisa kit (DS52021) from IBL International GmbH (Hamburg, Germany), following the instructions supplied by the manufacture.

Statistics

Statistical analysis was performed using SAS Enterprise Guide 4.3 statistical software (SAS Institute Inc, Cary, NC, USA) or Sigma Plot® 11.0 (Systat Software Inc, Chicago, IL, USA). The level of significance was set at 0.05. Data from continuous endpoints were examined for normal
distribution and homogeneity of variance and if necessary, were transformed. Organ weights were 
analysed using analysis of variance (ANOVA) with body weight as a covariate (ANCOVA) 
followed by a Dunnett post hoc test on all 4 groups. Body weights, onset of puberty, sperm counts 
and hormone data were analysed by ANOVA followed by a Dunnett post hoc test on all 4 groups. 
The statistical analyses of puberty and sperm counts were adjusted using litter as an independent, 
nested and/or random factor. Estrous cyclicity data were tested using logistic regression and tested 
for over dispersion with Deviance and Pearson Goodness-of-Fit tests and correction for over 
dispersion due to litter effects were used when appropriate. Furthermore, the data was analysed 
using the Cochran-Armitage trend test. Fisher’s exact test was used for statistical evaluation of 
histopathology and additionally Chi-square for trend was used for evaluation of epididymis 
histopathology.

Results

In vivo examinations and early death

There was no indication of general toxicity in dams or in the offspring at any of the administered 
doses. Details are presented in Christiansen et al. (2012).

Of the 208 weaned offspring, two rats died (one of them while anesthetized) and eight rats were 
sacrificed due to lack of general well-being such as weight loss and dullness or presence of large 
tumors before the planned necropsy, one at PD76 and all other when 12-19 months of age. These 
included two females from the control group, one male and two females from the TotalMix150 
group, three females from the TotalMix450 group and two females from the AAMix450 group. 
Four of these female offspring had pituitary adenomas and three females had mammary gland 
tumors.
The timing of male and female onset of puberty was not affected in any dose group compared to controls (data not shown).

No statistically significant differences between exposed groups and controls were found in the percentage of irregularly cycling females at 9 months of age (data not shown). At 12 months of age a trend towards increased frequency of irregularly cycling females was apparent in the groups dosed with TotalMix450 and AAMix450 (overall p-value = 0.07) (Fig.1). The trend was statistically significant when pooling data from the TotalMix450 and the AAMix450 group (p = 0.01) as was the comparison of the AAMix450 group with the controls (p < 0.05). In general, the female rats with irregular cycles had longer than normal cycles characterized by prolonged diestrus. This indicates that the female rats in the TotalMix450 and AAMix450 groups reached reproductive senescence earlier than control females, as increasing incidence of irregular cycles is a stage female rats pass through during the process of reproductive senescence (LeFevre & McClintock 1988).

**Pituitary tumors**

When pooling male and female data a statistically significant increased frequency in pituitary adenomas was seen in the AAMix450 group compared to the control group (Fig. 2). This difference was particularly marked and also statistically significant in males (57% (8 of 14) vs. 0% (0 of 12), respectively) (Fig. 2). For the females, a similar frequency (46% (6 of 13)) of pituitary adenomas was observed in the AAMix450 group, but this increased frequency was not statistically significant compared to controls (p = 0.07) (Fig. 2). As morphological separation of pituitary hyperplasia from adenomas can be difficult as these diseases probably represent a continuum during the transition from a normal pituitary gland to a neoplasm, the animals with hyperplasia and adenomas were also analysed as one group, and this revealed a statistically significant increase in rats with either pituitary adenoma or nodular hyperplasia in the TotalMix150 and the AAMix450 Mix groups (Fig. 2).
Pathology of aged female offspring

In aged female offspring statistically significant differences in body and organ weights were not observed between exposed groups and controls, although a tendency towards lower ovary weights was noted in the TotalMix450 group compared to controls ($p = 0.08$) (Table 2a).

No significant differences in ovarian and uterine histopathology or in estrus cycle stage were observed between exposed groups and controls (data not shown). Most females had several corpora lutea in the ovary and only one to two females from each high dose group showed absence of corpora lutea and presence of follicular cysts.

No dose-related trends were observed in the occurrence of mammary gland tumors, as these were observed macroscopically in three females belonging to the control group, two rats from the TotalMix150 group and three rats from the TotalMix450 group (when including three rats sacrificed before 19 months of age due to a mammary tumor). Female mammary glands with dilated ducts and with secretory material in the duct lumens were observed in 40% (4 of 10) of the rats in each of the control, TotalMix150 and TotalMix450 groups and in 55% (6 of 11) of rats in the AAMix450 group. These changes were related to the presence of pituitary adenomas, as they were seen in 91% (10 of 11) females with pituitary adenoma, but only in 30% (8 of 27) of females without gross lesions in the pituitary. These mammary gland changes are likely associated with hyperprolactinaemia caused by a hyperactive pituitary gland.

Sperm count and hormone levels

Epididymal sperm counts were significantly lower in all three dose groups compared to controls, when one outlier in the control group was excluded from the analysis ($p = 0.0004$, $p = 0.02$ and $p < 0.0001$, respectively) (Fig. 3). This outlier also had abnormal testis and epididymis, as described in the next section. When including the outlier in the analysis, the results showed no significant effects
in the TotalMix450 group, but significantly decreased numbers of sperm in the males exposed to TotalMix150 ($p=0.049$) and AAMix450 ($p < 0.0001$).

No significant effects were found on either testosterone or inhibin B hormone levels in males, and no correlations were seen between the two hormones or between these hormones and sperm count (data not shown).

Pathology of aged male offspring

No anomalies were observed in male external genitals at necropsy and no statistically significant differences between exposed groups and controls were observed for the body or organ weights in aged male offspring (Table 2b).

In the ventral prostate, a shift from the general age-related atrophy towards hyperplasia was observed following exposure to the contaminant mixture and to anti-androgens in particular. Higher scores for atypical hyperplasia (Fig. 4A) and a slight increase in the appearance of one to few small areas with cribriform patterns (Fig. 4B) were present in exposed groups compared to controls. The increase in atypical hyperplasia was statistically significant for the AAMix450 group. The increase in the occurrence of cribriform patterns was borderline significant in the TotalMix 150 group ($p = 0.054$) and appeared higher for TotalMix450 and AAMix 450, but was not statistically significant ($p=0.12$ in both groups). Furthermore, statistically significantly fewer acini with atrophic epithelium were found in rats in the AAMix450 group (Fig. 4C). Multifocal interstitial aggregations of inflammatory cells (score 2) were observed in 21% (3 of 14) of the rats in the TotalMix450 group and in 8% (1 of 13) of the rats in the AAMix450. In all other rats scattered interstitial inflammatory cells or focal to few multifocal interstitial aggregations of inflammatory cells (score 1) were observed. Severe chronic granulomatous inflammation was seen in the ventral prostate of one rat from the AAMix450 group, and this rat was excluded from further histological evaluation. Atypical
cells including intraepithelial vacuolations and variable amounts of intraluminal concretion were observed in the ventral prostate from all rats. A statistically significant increase in the incidence of high score for presence of concretion was seen in the AAMix450 group compared to the controls (Fig. 4D).

Histological examinations of the testes revealed general degeneration of seminiferous tubules only in the control animal that also had low sperm count, as this animal had degeneration of all tubules and a Sertoli cell only appearance. One animal from the TotalMix450 group had a low seminiferous epithelium and large lumina, but persisting spermatogenesis. Small areas of dysgenic tubules within Leydig cell clusters were observed in one to three males in all groups and this did not reach statistical significance in exposed groups relatively to controls (data not shown).

In the epididymis, several animals showed areas of epithelial vacuolization and degeneration combined with multilayering of epithelial cells with abnormal shapes and sizes (atypia). This finding was most frequent in animals from the TotalMix450 group, where 43% (6 of 14) of the animals had this phenotype. The same was seen in 23% (3 of 13) of the animals in the TotalMix150 group and in 14% (2 of 14) of the animals the AAMix450 group. In the control group, this finding was only seen in the animal which also had abnormal testes and a lack of spermatogenesis (1 of 13 (8%) animals). A chi-square test for trend comparing the control, TotalMix150 and TotalMix450 groups was statistically significant.

**Discussion**

In this study we revealed adverse effects on male and female offspring reproductive functions in later life stages as a result of developmental exposure to a human relevant mixture of environmental contaminants. As with the pre-pubertal effects in our earlier study (Christiansen et al. 2012) these
effects can most likely be attributed to the anti-androgenic compounds in the mixture. Estrogens in
the mixture did not seem to alter the effects of the anti-androgens neither by increasing nor
decreasing their magnitude. Most of the observed effects only reached statistical significance in the
AAMix450 group or in the TotalMix450 group, and generally similar effect patterns were seen in
these two groups. However, also in the TotalMix150 group indications of effects in ventral prostate,
pituitary gland and sperm count were observed in aged offspring, and in pre-pubertal rats increased
nipple retention was observed in this group (Christiansen et al. 2012). In regulatory studies, a
default uncertainty factor of 100 is commonly used for extrapolation of safe doses determined in rat
studies to doses that can be considered safe for humans (ECHA 2010). If effects of a compound are
seen at a LOAEL of 150 times human exposure levels, the safety margins would normally be
considered low and a sign of an insufficiently controlled risk. The same considerations will apply to
the current mixture, and in this case a safety margin of 100 may not be obtained for highly exposed
population groups. The indications of adverse effects at 150 times human exposure levels in this
study suggests that especially women of reproductive age may not be sufficiently protected against
combined effects of endocrine disrupting environmental chemicals.

The use of polycarbonated cages and water bottles, as in the present study, may have introduced a
background exposure to BPA (Howdeshell et al. 2003; Le et al. 2008; Duty et al. 2013) which
could have affected the biological endpoints. Background exposure to BPA or other possibly
endocrine disrupting chemicals present in water bottles or cages was not controlled in this study.
The doses of BPA present in the mixtures were between 0.225 mg/kg d (TotalMix150 group) and
0.675 mg/kg d (TotalMix450 group), far higher than the doses resulting from migration of BPA
water bottles into the drinking water estimated by Le et al. (2008). Based on these data, we estimate
(worst case) that migration from water bottles to drinking water might have resulted in BPA doses
of around 1% of those used in the mixtures, too low to make a measurable contribution in the
exposed groups. Possible variations in background exposure in control dams might have reduced the sensitivity of the study.

Reproductive aging in females and pituitary gland changes

The increased proportion of irregularly cycling females at 12 months of age in the high dose mixture groups is seen as a sign of premature reproductive senescence, as no differences from controls were seen at 9 months of age. This indicates that developmental exposure to a human relevant mixture of environmental contaminants advances reproductive senescence. This has previously been described by others for high doses of single chemicals (Armenti et al. 2008, Adewale et al. 2009). The process of follicle assembly and the recruitment of primordial follicles in the ovary to enter into growth are thought to be essential for the duration of the reproductive lifespan (Uzumcu & Zachow 2007, Crain et al. 2008, Diamanti-Kandarakis et al. 2009). Any chemical interfering with these processes may advance the onset of menopause. The hypothalamic-pituitary-ovarian axis is involved in the processes of reproductive aging in females, and reprogramming of the hypothalamic-pituitary-ovarian axis may also be involved in premature reproductive senescence (Gore et al. 2011). All chemicals included in the mixture have been shown to affect either estrogen or androgen actions, and some are capable of interfering with folliculogenesis or follicle health in the ovary (Crellin et al. 2001, Adewale et al. 2009, Rodríguez et al. 2010, Ahn et al. 2012, Wang et al. 2012, Zhang et al. 2013). Thus, direct or indirect mixture effects on the ovaries resulting in early reproductive aging are plausible. It was not possible to separate aging effects from treatment effects on reproductive senescence by histological examination of uterus and ovaries from the same rats at 19 months of age. Further examinations of ovarian follicles at other ages may clarify whether a reduction of follicle reserves or an accelerated rate of folliculogenesis may have caused the observed sign of premature reproductive senescence.
The observation of an increased incidence of pituitary adenomas in the mixture groups may be related to the altered age of reproductive senescence observed in the exposed females. Prolactin secretion increases with age, likely due to a reduction in hypothalamic dopamine activity, and a high incidence of prolactinomas is seen in old rats due to the lack of hypothalamic inhibitory control (Sarkar et al. 1982, Stefaneanu & Kovacs 1994). In rats, estrogen is shown to stimulate prolactin release from lactotrophs and inhibited the activity of hypothalamic neuroendocrine dopaminergic neurons, and prolactinomas can be induced in rats by prolonged administration of estrogen (Welsch et al. 1971, Sarkar et al. 1982, Lloyd 1990, DeMaria et al. 2000). Consequently, persistent changes in the androgen-estrogen balance in the mixture groups may have played a role in the development of pituitary tumors. Alternatively, administration of EDCs during critical periods of development can reprogram the hypothalamo-pituitary-gonadal axis and influence tumor development later in life. This may account for the observed changes in the timing of aging-related events in females. Although not immunohistochemically confirmed, a high number of the pituitary adenomas seen in the current studies are likely to be prolactinomas, as the observed mammary gland changes point to the presence of prolactinomas, and as prolactinomas are common in aging rats (Trouillas et al. 1982, Barsoum et al. 1985). The findings in the current study may be blurred by the lack of histological evaluation of female pituitaries without gross lesions. Thus, the actual number of tumors in females could be higher, as also indicated by mammary gland examination. As spontaneous pituitary adenomas are a common finding in aged rats the number of pituitary adenomas in the control groups in the current study may be unusually low (Trouillas et al. 1982, Barsoum et al. 1985, Carlus et al. 2013).

The current studies reveal important new knowledge about the influence of early endocrine disrupter exposure on reproductive aging in females, but it is not fully elucidated which parts of the hypothalamo-pituitary-gonadal axes are primarily responsive to early programming events.
Reproductive aging in males

The finding of reduced epididymal sperm counts in aged male offspring perinatally exposed to the contaminant mixture supported the hypothesis that perinatal exposure to endocrine disrupters can increase the risk of impaired fertility in humans. The magnitude of the risk for humans exposed to a similar chemical mixture cannot be determined, as the effect seen in the group exposed to the lowest dose of the mixture must be interpreted with caution. Results from this group were obtained from only three rats representing two litters, and analysis of the results from this group and the AAMix450 group was performed without simultaneous analysis of samples from the control group.

The observed epididymal changes in the high dose mixture group cannot readily be used as an explanation for sperm count reduction, but may rather be considered as a sign of a dose-related shift towards early aging of exposed males. The presence of dysgenetic tubules in testis in exposed groups is a finding that is commonly seen with exposure to certain phthalates including DEHP and DBP (Jarfelt et al. 2005, Mahood et al. 2007), and these effect are assumed to be related to the phthalate content of the mixture, but are not directly related to the sperm count reduction.

The ventral prostate normally atrophies with aging, and decreasing testosterone levels with age correlate with decreased weight of the ventral prostate (Isaacs 1984, Bosland, 1992, Lau et al. 2003). Interestingly, in the current studies a shift from the general age-related atrophy towards hyperplasia of the ventral prostate was observed following exposure to the contaminant mixture and to anti-androgens in particular. This conclusion is based on the presence of diminished epithelial atrophy, higher scores for atypical hyperplasia, and an increase in the appearance of cribriform patterns. Spontaneous tumors of the prostate are a rare finding in most rat strains (Ward et al. 1980, Reznik et al. 1981) including the Wistar strain (incidence 1% in 2-year studies, Poteracki & Walsh 1998) and therefore were not expected in the 19 months-old animals. Hyperplasia was thus
considered a marker of pre-cancerous lesions, and there is evidence that the atypical hyperplasia in the rat prostate may also progress to adenoma and carcinoma in some strains (Ward et al. 1980, Reznik et al. 1981, Isaacs 1984). In humans high-grade prostatic intraepithelial neoplasia (PIN)-lesions is considered as a likely precursor of prostatic adenocarcinoma. These lesions are characterized by cellular proliferations within preexisting ducts and acini with cytologic changes mimicking cancer (Bostwick et al. 2004). Consequently, this finding of increased hyperplasia may point to increased prostate cancer risk in humans following developmental exposure to the applied mixture and to anti-androgens in particular. Shorter anogenital distance in prostate cancer patients was recently shown in a human case-control study (Castaño-Vinyals et al. 2012). Developmental exposure to anti-androgens may play a role in prostate cancer development in men, as it is well recognized from animal studies that developmental exposure to anti-androgenic chemicals result in shorter anogenital distance in male rats (Hass et al. 2007, Christiansen et al. 2009). Anway and Skinner (2008) found an adult onset of mostly augmented atrophic changes and to a lesser extent hyperplastic changes to the aged ventral prostates through four generations following neonatal exposure to vinclozolin in F0 rats. Ho et al. (2006) and Prins et al. (2011) demonstrated that neonatal exposure to environmentally relevant doses of bisphenol A increased the susceptibility to precancerous prostate lesions in an experimental model where rats received supplemental sex steroids in adulthood. The exact mechanism behind these prostatic proliferative changes observed months after exposure is unknown, but epigenetic mechanism has be suggested to play a role (Ho et al. 2006, Anway & Skinner 2008).

**Conclusion**
The study showed that developmental exposure of rats to the highest dose of a human relevant environmental contaminant mixture of EDCs induced long-lasting effects manifested late in life. We found earlier reproductive senescence in the female offspring, reduced epididymal sperm count and a higher score for prostate atypical hyperplasia in male offspring as well as higher incidence of pituitary tumors in both sexes. These effects were mainly attributed to the anti-androgenic compounds in the mixture.

The observed changes in reproductive aging of females, in prostatic aging in males and in pituitary tumors would have been overlooked, if evaluations had only been performed in young adult animals as addressed in the current OECD guidelines for reproductive toxicology testing. In pre-pubertal male offspring from the same experiment, clear effects on early markers for endocrine disrupting effects such as increased incidence of nipple retention in all groups and reduced ventral prostate weight in high dose groups were shown (Christiansen et al. 2012). The effects found in the senescent animals emphasize that early findings of adverse effects of exposure can be a signal of severe adverse effects observed late in life.

Declaration of interest: This article is based on work presented at the 7th Copenhagen Workshop on Endocrine Disrupters, which was supported by the Danish Ministry of the Environment – Environmental Protection Agency. Publication of this special issue was supported by the Society for Reproduction and Fertility. The authors Louise Krag Isling, Julie Boberg, Pernille Rosenskjold Jacobsen, Karen Riiber Mandrup, Marta Axelstad, Sofie Christiansen, Anne Marie Vinggaard, Camilla Taxvig and Ulla Hass have a relationship with the Danish Ministry of the Environment-Environmental Protection Agency as they all give independent scientifically based advice to the Danish EPA. This is financed via an agreement between our Institute and the Danish EPA. Danish
EPA is a governmental institution and as such a non-profit organization. Our participation in COW was also financed by this funding from Danish EPA. Dr. Andreas Kortenkamp has no financial relationship with the Danish EPA. None of the authors have relationship with the Society for Reproduction and Fertility.

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Richard Sharpe (Edinburgh, UK)  In your rats exposed to the mixture of endocrine disrupter compounds (EDCs) did you look at hormone data? In the adult males was there any effect on the testosterone levels and the hormonal axis? Did you look at any aspects of metabolic function/disease to see if there were any changes in obesity or any other effects in addition to reproduction?

Ulla Hass (Søborg, Denmark)  We performed many hormone analysis but not testosterone in adult males although that might have been interesting. There was no significant effect on hormone levels in postnatal day 16 animals. This was a very large study and we were limited in the number of parameters we could measure. We did not look at the metabolic syndrome.

Hagai Levine (Jerusalem, Israel)  Telomere lengths are associated with late effects of many conditions. Did you look at telomere length in relation to EDC exposure?

Ulla Hass  That was not part of the study.

Laura Vandenberg (Medford, USA)  You performed a replicate nipple retention count on one study but not the other because you used a less sensitive method. The obvious way is to examine the underside and count the nipples with no obvious reason for inter observer variation.
Ulla Haas  We tried to train 2 technicians to count nipples the same way but it was not possible for them both to come to exactly the same answer. We were looking for areolas which are small dark spots and these are sometimes difficult to identify. In addition to the dark spots, small nipple buds can be felt on palpation of the skin, but dark spots and nipple buds are two different criteria for nipple retention and the 2 technicians were not always consistent in the criteria they used. Palpation was less sensitive than spot visualisation. For that reason, we decided to use the same technician throughout a single project for the sake of consistency.

Niels E Skakkebæk (Copenhagen, Denmark)  Your studies are important and should be promoted and repeated from a clinical point of view being designed to help protect humans. An effect is important if it causes disease in 5%, 10% or even 1 in a million of the human population.

Ulla Hass  We look for an effect in 20 experimental animals and try to extrapolate our findings to 600,000,000 people in the European Union. If we look for a yes/no answer as an effect on oestrus cyclicity we must see a large effect to reach significance such as an increase from 1% to 25% in irregular cycles. We are trying to protect all women, and not merely trying to find an exposure which will cause 25% of women to enter an early menopause. This must be borne in mind when assessing animal studies.

Andreas Kortenkamp (Uxbridge, UK)  I was alarmed by your prostate study showing evidence of atypical hyperplasia in animals exposed to an antiandrogen mix of chemicals and suggesting that this might be related to a risk of prostate cancer. How do you interpret this lesion?
Louise Krag Isling (Copenhagen, Denmark)  I am the pathologist who examined the prostates. There were lesions similar to atypical small acinar proliferation (ASAP) which is found in human prostates and is considered to be a possible pre-cancerous condition. We only saw early changes in the rats and follow up studies are required.

Ewa Rajpert-De Meyts (Copenhagen, Denmark)  The possibility of EDCs causing cancer is important. You are perhaps indicating that very early exposure during fetal development can initiate changes resulting in cancer in later life. Have you also looked at other tissues for histological abnormalities such as breast because female breast cancer is increasing dramatically worldwide and is attracting much attention?

Ulla Hass  We did not look at all of the organs in the body because this was not a cancer study. However, the mammary gland was examined although the data are not yet ready for publication. The rise in breast cancer incidence has been slowing in more recent publications.

Ewa Rajpert-De Meyts  Testicular cancer and germ cell tumours occur as a result of very early exposure, but rat is not a good model for these cancers. This study should be repeated on more susceptible animals such as mice taking into account those with and without genetic susceptibility. The 129 strain of mouse has a high incidence of testicular teratomas.
Ken Grigor (Edinburgh, UK)  There is evidence of reducing sperm counts and earlier puberty in humans. Is there evidence of any changes in the age of menopause?

Niels E Skakkebæk  There is no evidence of menopause generally occurring earlier.
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Figure legends

Figure 1:

The percent of regularly and irregularly cycling 12 months-old female offspring in the control, TotalMix150, TotalMix450 and AAMix450 group (N (offspring) = 13, 14, 14, 14, respectively). Overall p-value was 0.07, but statistically significant fewer regular cycling females were present in AAMix450 group when this group was compared to controls (* p < 0.05). The trend of increased incidence of irregularly cycling animals in exposed groups was statistically significant when pooling data from the TotalMix450 and the AAMix450 group (¤ p = 0.01). Logistic regression and Cochran-Armitage trend test used.

Figure 2:

Percent of male (M)# and female (F)# offspring in the control, TotalMix150, TotalMix450 and AAMix450 groups with adenoma (black columns) or focal nodular hyperplasia (grey columns) in the pars distalis of the pituitary gland. N (male offspring) =12, 11, 14, 14. N (female offspring) = 12, 13, 13, 13. Statistically significantly more males with pituitary adenoma were observed in the AAMix450 group compared to controls (see results section for more details). Fisher’s exact test used, ** p < 0.01, *** p < 0.001. Horizontal line (-) represent pooled male and female adenoma data.

# Four males from which the pituitary gland was not examined microscopically were omitted.

Figure 3:

Females killed before 19 months of age with gross lesions in the pituitary gland were included.

Only female pituitary glands with gross lesions were examined microscopically.
Number of sperm per g cauda in 19 month-old male rats in the control, TotalMix150, TotalMix450 or AAMix450 (N (offspring) = 13, 3, 13, 13, respectively). Individual male values are shown, together with group means and SEM. Statistically significantly fewer sperm per g cauda was observed in all exposed groups compared to control when analysed without the outlier in the control group (* p < 0.05) but only in the TotalMix150 and AAMix450 groups if the outlier was not excluded (¤ p < 0.05). Analysis of variance (ANOVA) test used.

Figure 4:

Results on ventral prostate histology (A-F) from 19 months-old male offspring are shown. Percentage of males in the control, TotalMix150, TotalMix450 and AAMix450 group (N (offspring) = 14, 13, 14, 13 with a given score in atypical hyperplasia (A), cribriform pattern (B), epithelial atrophy (C) and luminal concretion (D). The distribution of scores for atypical hyperplasia and epithelial atrophy were statistically significantly different between the AAMix450 group and the control group. Compared to controls, significantly more rats had atypical hyperplasia score 2 or 3 (pooled) and significantly more rats had epithelial atrophy score 1 in the AAMix450 group. Additionally, significant more rats in the AAMix450 group had luminal concretion score 3 compared to controls. Fisher’s exact test test used, * p < 0.05. E and F show histological sections of the ventral prostate from a control rat (E) with epithelial atrophy (arrows), and from a rat in the AAMix450 group (F) with atypical hyperplasia and cribriform pattern (arrows). Bar = 50 µm.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Purity % (CAS no.)</th>
<th>Adjusted intakes chosen as basis for mixture study (mg/kg d)</th>
<th>TotalMix (mg/kg d)</th>
<th>TotalMix (mg/kg d)</th>
<th>AAMix (mg/kg d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP*§</td>
<td>&gt;99.0 (84-74-2)</td>
<td>0.01 1.5 4.5 4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEHP*§</td>
<td>&gt;99.5 (117-81-7)</td>
<td>0.02 3 9 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinclozolin§</td>
<td>&gt;99.5 (50471-44-8)</td>
<td>0.009 1.35 4.05 4.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prochloraz§</td>
<td>&gt;98.5 (67747-09-5)</td>
<td>0.014 2.1 6.3 6.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procymidone§</td>
<td>&gt;99.5 (32809-16-8)</td>
<td>0.015 2.25 6.75 6.75</td>
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<td></td>
<td></td>
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<tr>
<td>Linuron§</td>
<td>&gt;99.0 (330-55-2)</td>
<td>0.0006 0.09 0.27 0.27</td>
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<td></td>
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<tr>
<td>Epoxiconazole§</td>
<td>&gt;99.0 (106325-08-8)</td>
<td>0.01 1.5 4.5 4.5</td>
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<td></td>
<td></td>
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<tr>
<td>p,p'-DDE§</td>
<td>&gt;98.5 (72-55-9)</td>
<td>0.001 0.15 0.45 0.45</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4-MBC*</td>
<td>&gt;98.0 (36861-47-9)</td>
<td>0.06 9 27 0</td>
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<td></td>
<td></td>
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<tr>
<td>OMC§</td>
<td>&gt;98.0 (5466-77-3)</td>
<td>0.12 18 54 0</td>
<td></td>
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<tr>
<td>Bisphenol A*</td>
<td>&gt;99.5 (80-05-7)</td>
<td>0.0015 0.225 0.675 0</td>
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<tr>
<td>Butyl paraben*</td>
<td>&gt;99.0 (94-26-8)</td>
<td>0.06 9 27 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracetamol§</td>
<td>&gt;99.0 (103-90-2)</td>
<td>0.8 120 360 360</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sum (mg/kg d)</strong></td>
<td></td>
<td>1.12 168 504 396</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Christiansen et al. 2012 for estimates of high end human intakes and for the adjusted intakes which were chosen as basis for the mixture study.*DBP (di-n-butyl phthalate), DEHP (di-(2-ethylhexyl) phthalate), p,p′-DDE (dichlorodiphenyldichloroethylene), 4-MBC (4-methyl-benzylidene camphor), OMC (octyl methoxycinnamate). § Purchased from VWR - Bie & Berntsen (Herlev, Denmark). * Purchased from Sigma-Aldrich (Brøndby, Denmark). § Mixtures contained paracetamol on GD 13-19.
Table 2a: Body and organ weights (g) 19 months-old perinatally exposed female rats

<table>
<thead>
<tr>
<th>Mixture (dose in mg/kg bw/day)</th>
<th>N (n)</th>
<th>Body weight</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 (11)</td>
<td>333 ± 38</td>
<td>0.692 ± 0.173</td>
<td>0.042 ± 0.0106</td>
<td>8.22 ± 0.98</td>
</tr>
<tr>
<td>Mixture of 13 anti-androgenic and estrogenic compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotalMix150 (168.12)</td>
<td>12 (11)</td>
<td>341 ± 30</td>
<td>0.721 ± 0.229</td>
<td>0.042 ± 0.009</td>
<td>8.33 ± 0.76</td>
</tr>
<tr>
<td>TotalMix450 (504.50)</td>
<td>11 (11)</td>
<td>358 ± 57</td>
<td>0.852 ± 0.371</td>
<td>0.037 ± 0.009</td>
<td>8.51 ± 1.15</td>
</tr>
<tr>
<td>Mixture of 9 anti-androgenic compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAMix450 (395.82)</td>
<td>12 (12)</td>
<td>326 ± 47</td>
<td>0.651 ± 0.202</td>
<td>0.043 ± 0.011</td>
<td>7.68 ± 1.19</td>
</tr>
</tbody>
</table>

Data represent means ± SD. N = number of offspring, n = number of litters. No statistically significant changes in body or organ weights were detected.
Table 2b: Body and organ weights (g) from 19 months-old perinatally exposed male rats

<table>
<thead>
<tr>
<th>Mixture (dose in mg/kg bw/day)</th>
<th>N (n)</th>
<th>Body weight</th>
<th>Pooled testes</th>
<th>Epididymides</th>
<th>Ventral prostate</th>
<th>Seminal vesicle</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14 (13)</td>
<td>584 ± 48</td>
<td>4.14 ± 0.81</td>
<td>0.736 ± 0.078</td>
<td>0.587 ± 0.153</td>
<td>1.74 ± 0.37</td>
<td>15.6 ± 2.15</td>
</tr>
<tr>
<td><strong>Mixture of 13 anti-androgenic and estrogenic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TotalMix150 (168.12)</td>
<td>13 (12)</td>
<td>585 ± 70</td>
<td>4.00 ± 0.68</td>
<td>0.708 ± 0.100</td>
<td>0.644 ± 0.162</td>
<td>1.80 ± 0.24</td>
<td>14.7 ± 1.70</td>
</tr>
<tr>
<td>TotalMix450 (504.50)</td>
<td>14 (13)</td>
<td>561 ± 51</td>
<td>4.27 ± 0.56</td>
<td>0.720 ± 0.057</td>
<td>0.673 ± 0.144</td>
<td>2.06 ± 0.74</td>
<td>14.4 ± 1.88</td>
</tr>
<tr>
<td><strong>Mixture of 9 anti-androgenic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAMix450 (395.82)</td>
<td>14 (14)</td>
<td>586 ± 77</td>
<td>4.25 ± 0.32</td>
<td>0.720 ± 0.044</td>
<td>0.712 ± 0.193</td>
<td>2.04 ± 0.46</td>
<td>15.1 ± 2.08</td>
</tr>
</tbody>
</table>

Data represent means ± SD. N = number of offspring, n = number of litters. No statistically significant changes in body or organ weights were detected.
The percent of regularly and irregularly cycling 12 months-old female offspring in the control, TotalMix150, TotalMix450 and AAMix450 group (N (offspring) = 13, 14, 14, 14, respectively). Overall p-value was 0.07, but statistically significant fewer regular cycling females were present in AAMix450 group when this group was compared to controls (* p < 0.05). The trend of increased incidence of irregularly cycling animals in exposed groups was statistically significant when pooling data from the TotalMix450 and the AAMix450 group (* p = 0.01). Logistic regression and Cochran-Armitage trend test used.

80x49mm (150 x 150 DPI)
Percent of male (M)# (blue columns) and female (F)# (red columns) offspring in the control, TotalMix150, TotalMix450 and AAMix450 groups with adenoma (dark color) or focal nodular hyperplasia (light color) in the pars distalis of the pituitary gland. N (male offspring) = 12, 11, 14, 14. N (female offspring) = 12, 13, 13, 13. Statistically significantly more males with pituitary adenoma were observed in the AAMix450 group compared to controls (see results section for more details). Fisher’s exact test used, ** p < 0.01, *** p < 0.001. Horizontal line (-) represent pooled male and female adenoma data.

# Four males from which the pituitary gland was not examined microscopically were omitted. Females killed before 19 months of age with gross lesions in the pituitary gland were included. Only female pituitary glands with gross lesions were examined microscopically.

119x89mm (300 x 300 DPI)
Number of sperm per g cauda in 19 month-old male rats in the control, TotalMix150, TotalMix450 or AAMix450 (N (offspring) = 13, 3, 13, 13, respectively). Individual male values are shown, together with group means and SEM. Statistically significantly fewer sperm per g cauda was observed in all exposed groups compared to control when analysed without the outlier in the control group (* p < 0.05) but only in the TotalMix150 and AAMix450 groups if the outlier was not excluded (≠ p < 0.05). Analysis of variance (ANOVA) test used.
Results on ventral prostate histology (A-F) from 19 months-old male offspring are shown. Percentage of males in the control, TotalMix150, TotalMix450 and AAMix450 group (N (offspring) = 14, 13, 14, 13 with a given score in atypical hyperplasia (A), cribriform pattern (B), epithelial atrophy (C) and luminal concretion (D). The distribution of scores for atypical hyperplasia and epithelial atrophy were statistically significantly different between the AAMix450 group and the control group. Compared to controls, significantly more rats had atypical hyperplasia score 2 or 3 (pooled) and significantly more rats had epithelial atrophy score 1 in the AAMix450 group. Additionally, significant more rats in the AAMix450 group had luminal concretion score 3 compared to controls. Fisher’s exact test test used, * p < 0.05. E and F show histological sections of the ventral prostate from a control rat (E) with epithelial atrophy (arrows), and from a rat in the AAMix450 group (F) with atypical hyperplasia and cribriform pattern (arrows). Bar = 50 µm.

205x215mm (300 x 300 DPI)